

Monocyte Recruitment and Expression of Monocyte Chemoattractant Protein-1 Are Developmentally Regulated in Remodeling Bone in the Mouse

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Tooth eruption is defined as the movement of a tooth from its site of development within the alveolar bone to its position of function in the oral cavity. It represents an excellent model to examine osseous metabolism as bone resorption and bone formation occur simultaneously and are spatially separated. Bone resorption occurs in the coronal (occlusal) area, whereas bone formation occurs in the basal area. Monocytes are thought to have a significant role in the regulation of osseous metabolism. The goal of this study was to examine the recruitment of monocytes to bone in C57BL/6J mice that are undergoing developmentally regulated bone remodeling. Monocytes were detected by immunohistochemistry and osteoclasts were counted as bone-associated multi-nucleated, tartrate-resistant acid phosphatase (TRAP)-positive cells. Cell numbers were obtained from histological sections of animals sacrificed daily for 14 days after birth; an image analysis system was used for quantification. The results demonstrated that, immediately after birth, there were relatively few monocytic cells. In the area of bone resorption, the number of monocytes increased with time, reaching peaks at 5 and 9 days, and decreased thereafter. A similar pattern was observed for osteoclasts. In the area of bone formation, there was a time-dependent increase in the number of monocytes. In contrast, the number of osteoclasts in this area was highest at the earliest time points and decreased after day 3. To investigate potential

mechanisms for the recruitment of monocytes, expression of monocyte chemoattractant protein (MCP)-1 was assessed. The number of MCP-1-positive cells increased with time and was generally proportional to the recruitment of mononuclear phagocytes. Osteoblasts were the principal bone cell type expressing MCP-1. The results demonstrate that the recruitment of mononuclear cells in the occlusal area is associated with bone resorption. In contrast, recruitment of monocytes in the basal area is associated with bone formation and a decrease in the number of osteoclasts. These results suggest that monocytes have different functional roles in areas of bone formation compared with bone resorption. Furthermore, the expression of MCP-1 is developmentally regulated and may provide a mechanistic basis to explain the recruitment of monocytic cells. (Am J Pathol 1997, 150: 1711–1721)

There are several lines of evidence that monocyte recruitment is important in tissue reorganization associated with development. Monocyte recruitment occurs at specific time points in early development of hematopoietic tissue,¹ in the developing diaphragm,² in the developing embryonic avian central nervous system,³ and in interdigital spaces during development of mouse limbs.⁴ To date, little has been reported concerning the expression of mediators that could account for the specific recruitment of monocytes during development.

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The classic leukocyte chemoattractants cannot account for the recruitment of specific leukocytes observed during development and chronic inflammation. Chemokines, members of the cytokine family of regulatory proteins, are chemotactic for specific leukocyte subsets. Monocyte chemoattractant protein (MCP)-1 is a chemokine that stimulates chemotaxis of monocytes *in vitro* and *in vivo*, is produced locally by various cell types, and is thought to be an important regulator of monocyte recruitment in a number of pathological conditions.^{5–11} Under most normal conditions, MCP-1 is not constitutively expressed but is induced by inflammatory conditions.^{12–14} For example, we have reported that in normal bone MCP-1 is not expressed, whereas its expression is stimulated in osteoblasts by experimentally induced osseous inflammation.⁷ Furthermore, MCP-1 production temporally and spatially coincides with recruitment of monocytes in this model.

Tooth eruption provides an excellent model to examine developmental changes that involve the coordinated cellular activities in both bone formation and resorption.^{15,16} Tooth eruption is defined as a migration of a tooth from its developmental site in the jaw bones to its functional position in the oral cavity.¹⁷ The period during which tooth eruption occurs differs in various species: 2 weeks for rodent molars compared with several months for human teeth.¹⁸ Until it emerges into the oral cavity, a developing tooth is surrounded by the dental follicle, a loose connective tissue sac that has multiple functions (see Figure 1). For a tooth to erupt, bone resorption occurs in the occlusal area along the path of eruption and bone formation occurs in the basal area on the opposite side.^{19–22} The cellular activity in the basal area differs considerably from that in the occlusal and is characterized by tissue remodeling and variable degrees of bone formation with little or no bone resorption.²¹ Despite many reports on this complex, multi-stage process, the exact mechanisms responsible for tooth eruption are still not fully understood.

Cells of the monocytic lineage are thought to play an important role in bone metabolism during tooth eruption. An influx of monocytes at the occlusal aspect of the dental follicle has been described 3 days postnatally in rats.^{23,24} This increased recruitment of monocytes to the dental follicle was correlated with changes in the number of osteoclasts that formed.²¹ The importance of monocytic cells in tooth eruption was further underscored by findings that the normal pattern of tooth eruption is dependent on production of colony-stimulating factor-1.^{23,25} Although there is a considerable documentation of monocytic cell infiltration of the dental follicle, little has been reported

on the recruitment of monocytes to bone during the developmental changes of tooth eruption.

In studies described below, we investigated the recruitment of monocytic cells to areas of developmentally regulated bone formation and bone resorption. To gain insight into the mechanisms potentially responsible for this recruitment, we examined the distribution of MCP-1-positive cells at different time points during tooth eruption. The results indicate that the expression of MCP-1 is developmentally regulated and is temporally linked to developmental changes in remodeling tissue. They also demonstrate that enhanced monocyte recruitment occurs in areas of bone resorption and, surprisingly, in areas of bone formation. In bone-resorbing areas, recruitment of cells of the monocytic lineage is associated with formation of osteoclasts. In bone-forming areas, monocyte recruitment is not associated with osteoclast formation. This suggests that monocytes recruited to each area are functionally distinct as they are associated with considerably different cellular processes.

Materials and Methods

Fifteen different litters of C57BL/6J newborn mice were studied in the experiments described below. Mice were sacrificed by carbon dioxide overdose and decapitated on days 0 to 14 after birth. Mandibles were immediately resected and immersed for 4 hours at 4°C in periodate-lysine-paraformaldehyde, as described by McLean and Nakane.²⁶ After fixation, samples were consecutively washed in 5, 10, and 15% glycerol in phosphate-buffered saline for 15 minutes each at 4°C. Specimens were then decalcified in 15% glycerol/EDTA for 2 to 4 days as we have previously described.⁷ Samples were then incubated in 30% sucrose overnight at 4°C to decrease freezing artifacts, snap-frozen by immersion in pre-chilled (–80°C) 2-methylbutane, and stored at –80°C.

There were three animals per time point. From each animal, two first molars and adjacent tissue were prepared for cryostat sections resulting in a total of six specimens per time point. For each specimen, immunohistochemistry was carried out using several different antibodies. A minimum of three histological sections were used for each antibody per specimen to ensure consistency of results.

Immunohistochemistry

Frontal sections were prepared perpendicular to the occlusal surface of the first mandibular molar. This

allowed an examination of bone adjacent to occlusal surface and in the basal area between the fornix and the developing apical area. Care was taken to ensure that the same region of each specimen was analyzed. Frozen serial sections (5 to 6 μm) were incubated with a specific polyclonal antibody (1:500 dilution) raised against murine MCP-1, which was generously donated by Dr. Barrett Rollins.^{27,28} As a negative control, sections from adjacent areas were incubated with nonimmunized normal rabbit serum or with hyperimmune rabbit serum raised against an irrelevant protein, human histatin. The rat monoclonal antibody F4/80, which identifies a plasma membrane marker on murine monocytes and macrophages was used to identify mononuclear phagocytes.^{29,30} Sections were incubated with secondary biotinylated antibodies and then incubated with 0.3% hydrogen peroxide in methanol to inhibit endogenous peroxidase activity; however, suppression was not 100% in a few hematopoietic cells in the bone marrow. Antibodies were localized by an indirect immunoperoxidase technique (avidin-biotin-horseradish peroxidase complex) employing diaminobenzidine as a chromogen (Vector Laboratories, Burlingame, CA). Slides were counterstained with hematoxylin.

Tartrate-Resistant Acid Phosphatase (TRAP) Assay

TRAP staining was used to detect and quantify multi- and mononuclear TRAP-positive cells lining bone, following the procedure described by Minkin.³¹

Quantitative Analysis

Three sections per antibody per specimen were analyzed. Tissue specimens were examined at $\times 400$ magnification, and cells were counted on a system that used an RGB video camera and microscope with the projected image analyzed by Image ProPlus software. The number of MCP-1-, F4/80-, and TRAP-positive cells in areas of bone resorption and formation were evaluated in two areas: 1) the occlusal area between the dental follicle and bone and 2) the basal area between the first molar apex and bone. Cell counts were expressed as the number of cells per tissue area (cells/mm²).

Statistical Analysis

Analysis of variance was performed to detect a difference in the number of cells from day 0 to day 14 in the occlusal and basal areas. A Student-Newman-

Keuls test was used to establish statistical significance of the quantitative changes that occurred between day 0 and day 14 for F4/80-positive and MCP-1-positive cells. A Pearson's correlation coefficient test was performed between the following parameters: 1) F4/80-positive cells and MCP-1-positive cells, 2) F4/80-positive and TRAP-positive cells, and 3) MCP-1-positive and TRAP-positive cells. Analyses were performed separately for the occlusal area (bone resorption) and basal area (bone formation).

Results

Figure 1 represents the developing tooth and associated bone. A tooth develops within its follicle and is surrounded by bone. At birth, the tooth consists of only a crown; root formation starts after day 5. The cells of the follicle will form a periodontal ligament when the tooth emerges into the oral cavity, attaching the tooth to alveolar bone. Within a few days after birth, marrow spaces become evident and enlarge over 7 to 10 days. Thus, the principal tissue changes that occur during tooth eruption involve resorption of bone and connective tissue overlying the tooth, root development, formation of periodontal ligament, and formation and remodeling of alveolar bone in the basal area of the tooth.

In the first set of experiments, monocyte recruitment was examined simultaneously with identification of MCP-1-positive cells. Monocytes were quantified by immunohistochemistry using the monoclonal antibody F4/80.^{29,30} Serial sections were also immunostained with specific antiserum to murine MCP-1 to identify and quantify the number of MCP-1-positive cells. This analysis allowed us to compare the temporal and spatial distribution of MCP-1 in the context of monocyte recruitment. All quantitative changes in MCP-1 and F4/80 expression were measured relative to day 0. In Figure 2, a 9-day specimen was immunostained with F4/80 and MCP-1 antibodies. Day 9 was chosen because it represents a midpoint in tooth eruption during which there are significant changes in the occlusal area associated with bone resorption. Considerable monocyte infiltration is present (Figure 2, A and B). The immunostaining is specific as shown by the absence of stained cells in sections incubated with the negative control, normal rat IgG (Figure 2C). The greatest number of monocytes are located between the follicle and the bone (Figure 2, A and B). Considerable expression of MCP-1 was detected at the same time point using an antibody to MCP-1 (Figure 2, D and E), which was specific as determined by lack of immunostaining with the negative control (Figure 2F). MCP-1-

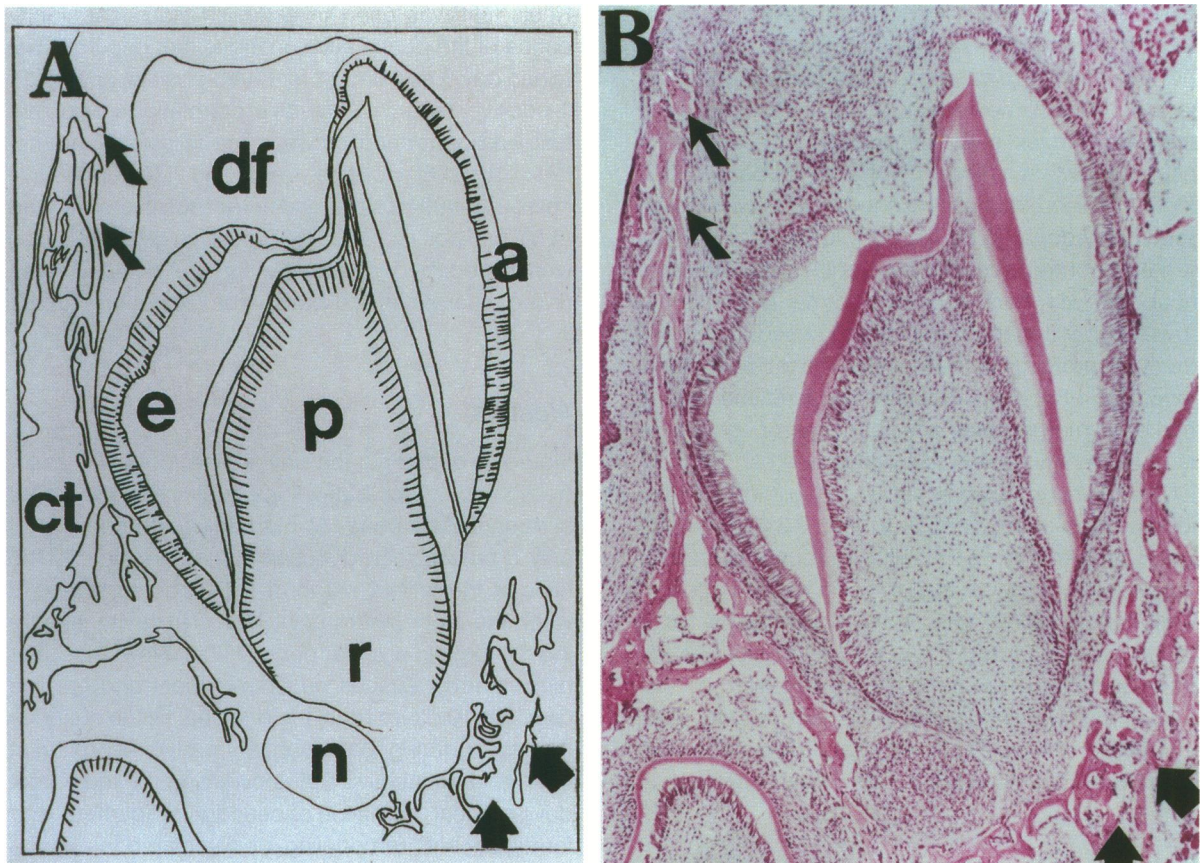


Figure 1. Histological features of the mouse first molar at day 9 postpartum. Tissues were then fixed, decalcified, sectioned, and stained with hematoxylin and eosin (H&E). **A:** Schematic representation of the first mouse molar at day 9. df, dental follicle (a loose connective tissue sac surrounding a tooth during its development); e, enamel space (enamel that has been decalcified during tissue preparation); a, ameloblasts (enamel-producing cells of ectodermal origin); p, pulp; r, root; n, nerve; ct, connective tissue. The short arrows indicate areas of bone formation in the basal portion (thick arrows) and bone resorption in the occlusal portion (thinner arrows) of the bone surrounding the tooth. **B:** H&E-stained sections of the first molar at day 9. The short arrows indicate areas of bone formation in the basal portion (thick arrows) and bone resorption in the occlusal portion (thinner arrows) of the bone surrounding the tooth.

positive cells associated with bone were osteoblasts. Other cell types that expressed MCP-1 were ameloblasts and gingival epithelium (recruitment of monocytes to close proximity of these cells was also observed; data not shown). The expression of MCP-1 by cells of epithelial origin (ameloblasts) was not unexpected given its constitutive expression by epithelial cells in skin.^{8,11} These cells were also negative when incubated with a control serum.

A more detailed analysis of MCP-1 expression in the area of bone resorption is shown in Figure 3. At day 1, there are few MCP-1-positive cells associated with bone (Figure 3A). On day 9, there is a large increase in the number of MCP-1-positive cells, which is consistent with osteoblasts lining bone (Figure 3D). Also evident is the expression of MCP-1 by fibroblastic cells in the connective tissue surrounding bone. Concomitant with the increase in MCP-1-positive cells is a substantial increase in the number of monocytes located between the dental follicle and

bone (Figure 3, B and E). A substantial increase in osteoclasts also occurred in the same time frame; there were few TRAP-positive osteoclasts on day 1 (Figure 3C) and a much greater number on day 9 (Figure 3F). This gives insight into the physiological state of bone as the number of osteoclasts is directly proportional to the degree of resorptive activity.

An analysis of the area of bone formation is shown in Figure 4. From day 1 to day 9, there is a substantial increase in the number of MCP-1-positive cells (Figure 4, A and D) and the number of monocytes (Figure 4, B and E). The largest increase in MCP-1 is seen in bone-lining osteoblasts, and the largest increase in monocytes occurs in close proximity to bone (Figure 4, D and E). In some cases, there is apparent immunostaining of cells in the bone marrow (Figure 4, D and E). This is nonspecific and is likely due to high levels of endogenous peroxidase activity in some cells in the bone marrow. Osteoclasts present in the basal area on day 1 are shown in

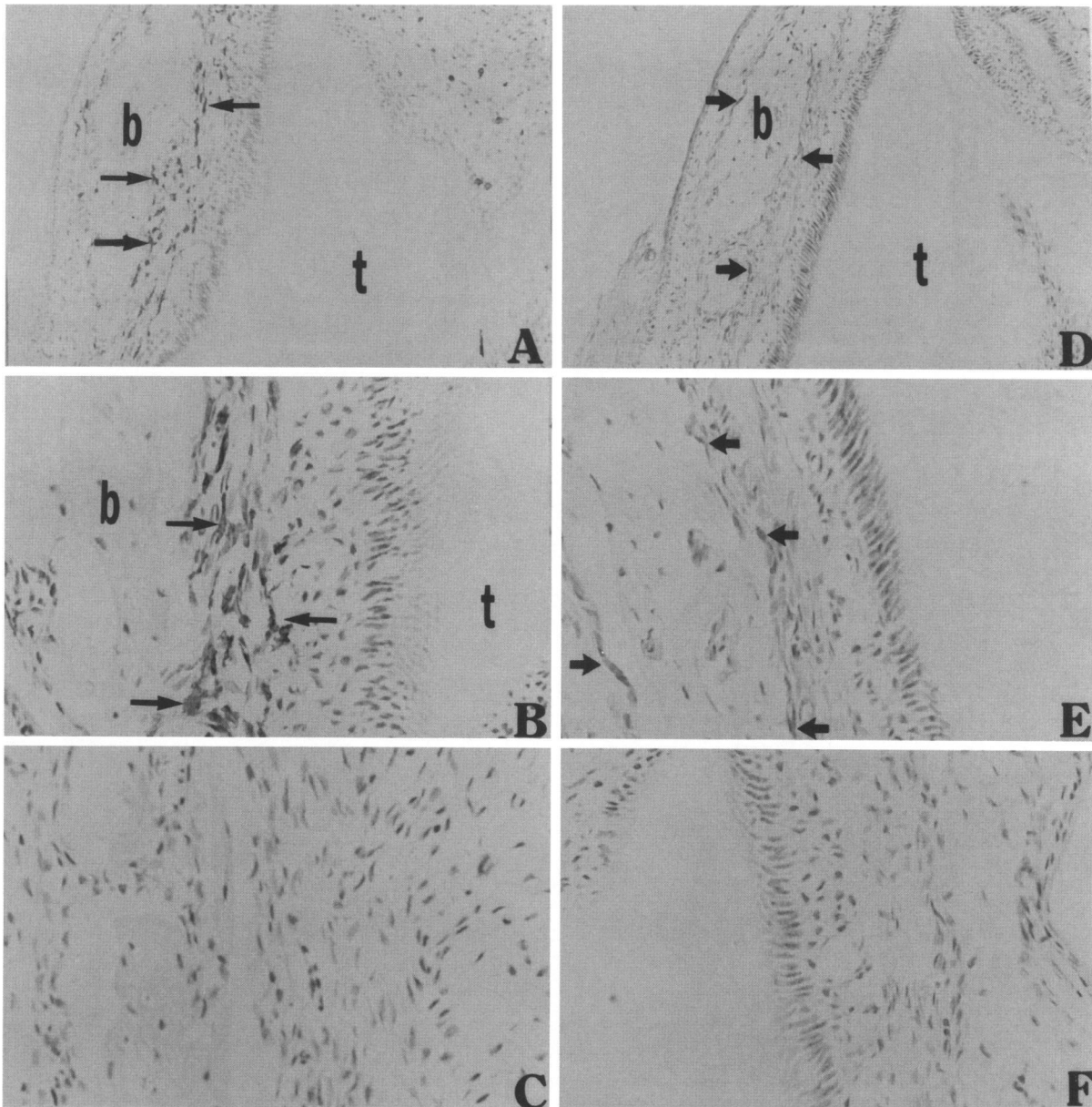


Figure 2. Distribution of mononuclear phagocytes and MCP-1-positive cells in the occlusal area at day 9. Cells were identified by incubation of cryostat-prepared sections with F4/80 monoclonal antibody to murine monocytes (**A** and **B**), control normal rat IgG (**C**), antibody specific for murine MCP-1 (**D** and **E**), or control rabbit hyperimmune serum against irrelevant antigen (**F**). Arrows indicate F4/80-positive cells and MCP-1-positive cells. b, bone; t, tooth. Original magnification, $\times 200$ or $\times 500$.

Figure 4C. In contrast to what was observed in the occlusal area, there was a decrease in the number of osteoclasts by day 9 (Figure 4F), indicating a trend toward little or no bone resorption.

Quantitation of Cellular Changes in Areas of Bone Resorption

To quantify changes in areas of bone resorption, the number of monocytes, MCP-1-positive cells, and osteoclasts were counted in the occlusal area in mice

sacrificed daily from day 0 to day 14 (Figure 5). The greatest increase in the number of MCP-1-positive cells in the occlusal area occurred on days 5 and 8 (Figure 5B). The greatest influx of monocytes occurred at days 5 and 9, showing a 2-fold and 2.5-fold increase, respectively (Figure 5C). After day 10, a significant decrease in MCP-1-expressing cells was observed. This decrease likely results from a net loss of osteoblasts associated with net bone resorption. With less bone surface, there would be fewer MCP-1-positive osteoblasts. The number of osteoclasts

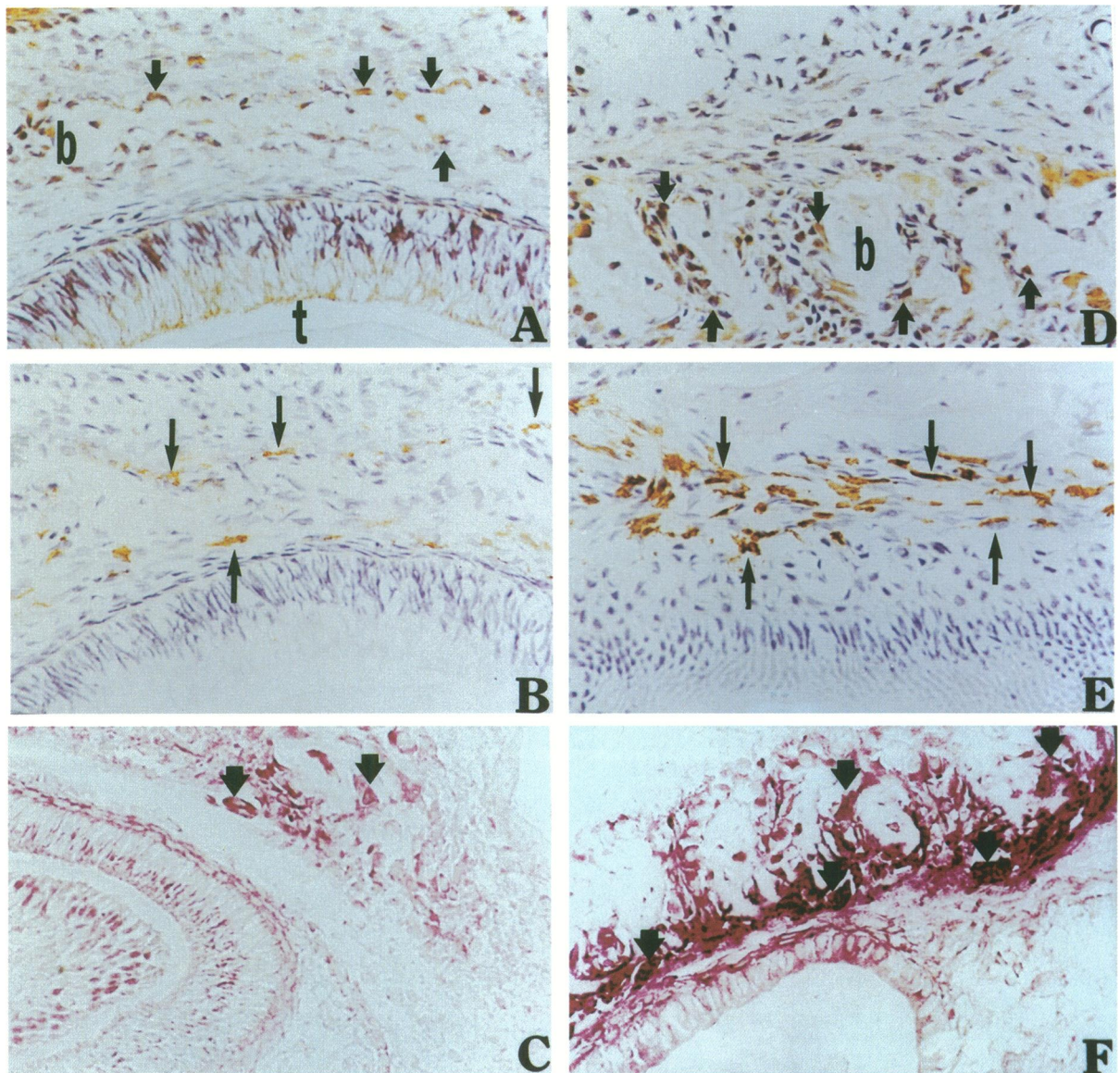


Figure 3. MCP-1 expression, monocyte recruitment and osteoclast distribution in areas of bone resorption. Immunohistochemistry on frozen sections was carried out as described in Materials and Methods using a specific antibody to murine MCP-1 and the F4/80 antibody to murine mononuclear phagocytes. Tartrate-resistant acid phosphatase staining was performed on cryostat-prepared sections. Each row represents sections stained for MCP-1 (A and D), F4/80 (B and E), and TRAP (C and F). Mice were sacrificed on day 1 (A to C) and day 9 (D to F). Arrows indicate F4/80-positive monocytes in close proximity to bone surfaces (B and E) or MCP-1 positive osteoblastic cells lining bone (A and D). Arrows in C and F point at osteoclastic cells on the bone surface at the occlusal area. b, bone; t, tooth. Original magnification, $\times 500$.

increased continuously from day 1 to day 12 (Figure 5A). A statistically significant 3-fold increase was noted at day 6 and a 6.5-fold increase at day 12. After day 12, the number decreased due to the decrease in bone surface area. The number of MCP-1-positive cells was temporally related and proportional to the recruitment of monocytes, as shown by Pearson's correlation test ($r = 0.76$; $P < 0.01$). Statistical analysis showed a positive correlation between the recruitment of monocytes and the number of osteoclasts in the occlusal area ($r = 0.55$; $P < 0.05$).

Quantitation of Cellular Changes in Areas of Bone Formation

Even though there are clear differences in osseous metabolism in the occlusal and basal areas of the erupting tooth, similar peaks in monocyte recruitment and the number of MCP-1-positive cells were observed for both areas. For monocytes, two peaks representing a 5-fold increase occurred on days 5 and 9 (Figure 5F). Two peaks of MCP-1 expression were also detected at the same time points, repre-

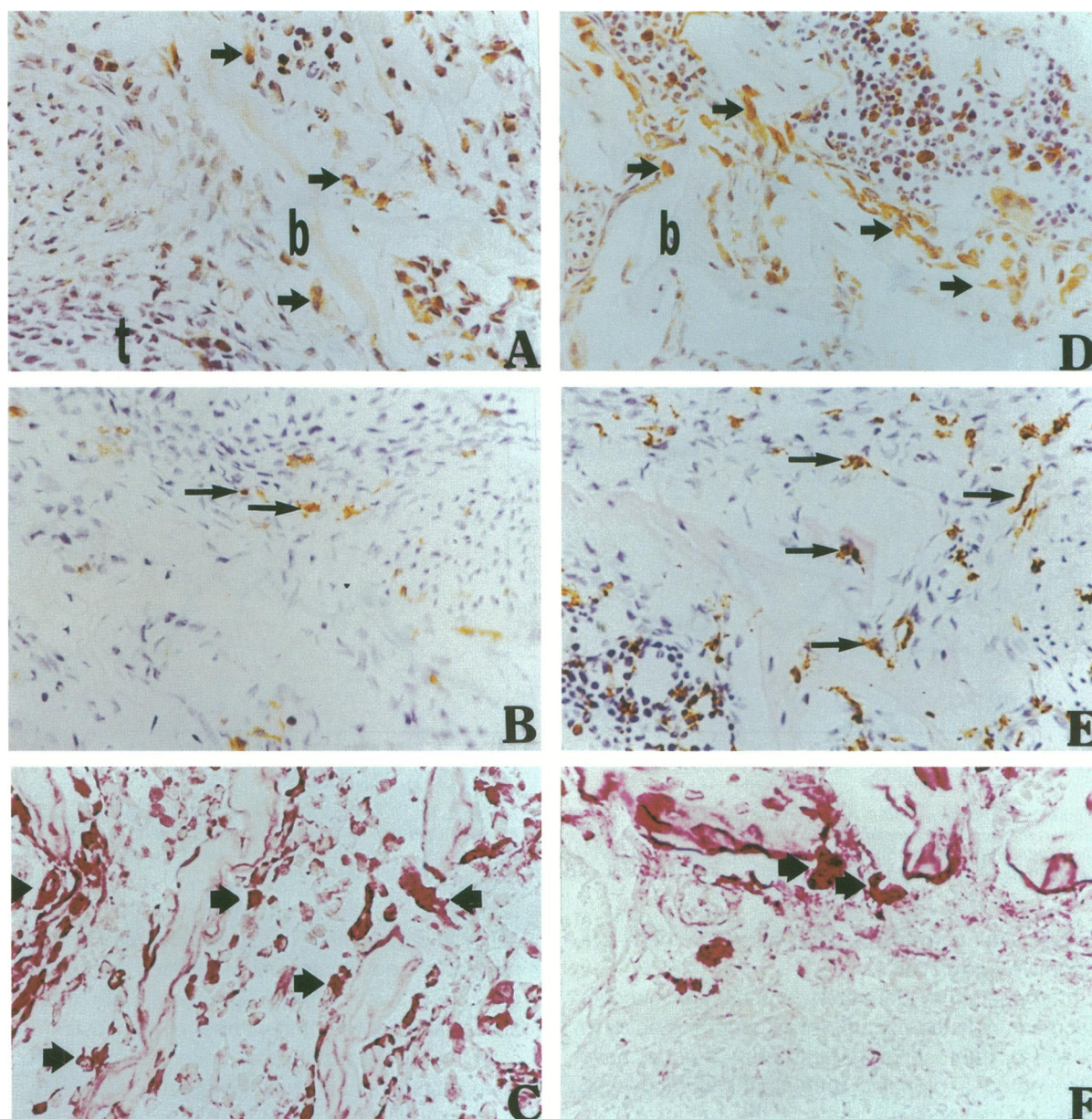


Figure 4. MCP-1 expression, monocyte recruitment, and distribution of osteoclasts in areas of bone formation. Immunohistochemistry and TRAP was performed as described in the legend of Figure 3. Each row represents sections stained for MCP-1 (A and D), F4/80 (B and E), and TRAP (C and F). Mice were sacrificed on day 1 (A to C) and day 9 (D to F). Arrows indicate F4/80-positive monocytes in close proximity to bone surfaces (b) or MCP-1-positive osteoblastic cells lining bone. b, bone; t, tooth. Original magnification, $\times 500$.

senting a 2.5-fold increase on day 5 and a 2-fold increase on day 9 (Figure 5E). After day 9, a consistent decrease in the number of MCP-1-positive cells was observed. The decrease in MCP-1 cannot be explained by a decrease in the bone surface area and, hence, is likely to directly represent decreased expression associated with developmental changes. The number of osteoclasts was highest at the initial time points and consistently decreased after day 3 (Figure 5D).

The number of MCP-1-positive cells was statistically correlated with the recruitment of monocytes as determined by Pearson's correlation test ($r = 0.548$; $P < 0.05$). For the basal area, there was an inverse relationship between the number of monocytes and osteoclasts ($r = -0.48$; $P = 0.05$). Thus, a striking difference is observed between areas of bone resorption and bone formation. In areas of resorption there is a positive correlation between monocyte recruitment and the number of

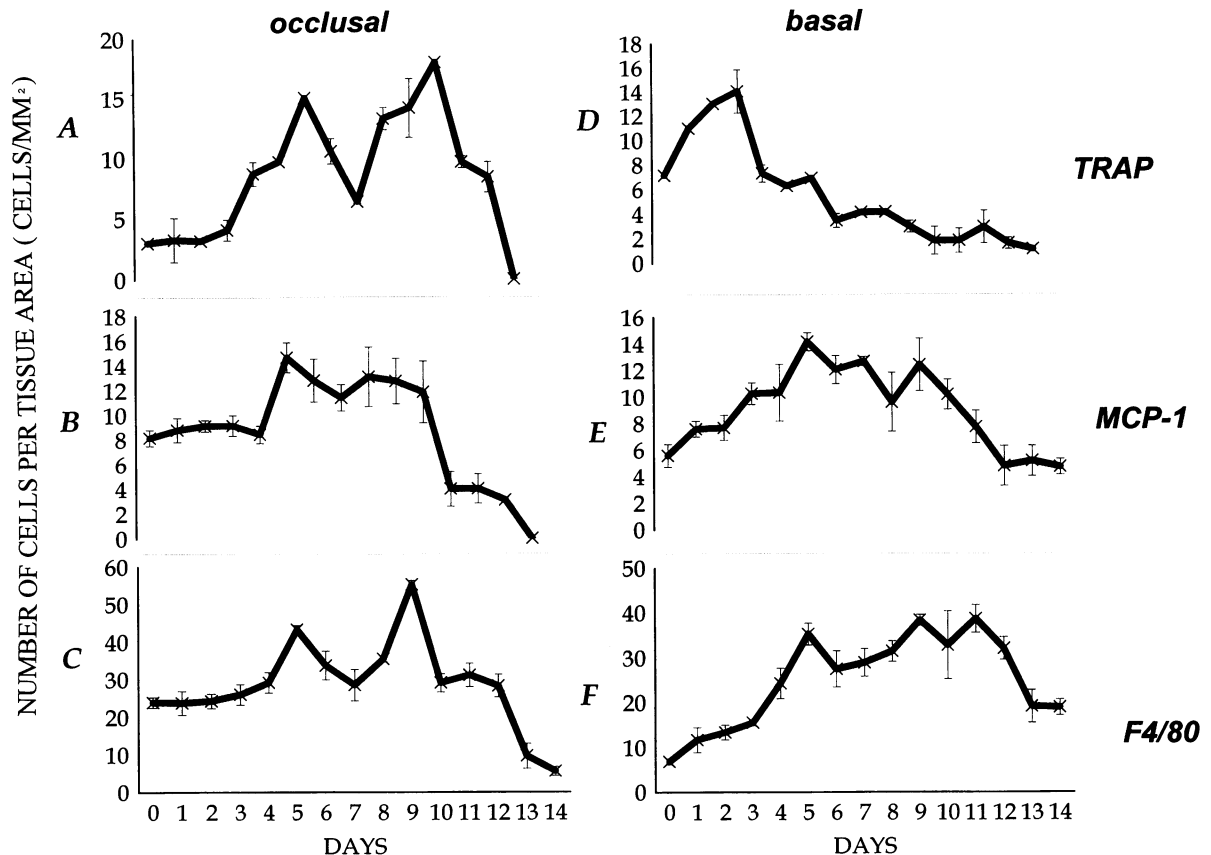


Figure 5. Quantitation of MCP-1-positive cells, monocytes, and osteoclasts per unit area. Mice were killed from birth to day 14 postpartum. Adjacent sections showing the first molar and the surrounding bony crypt were used to identify MCP-1 with an antibody specific for MCP-1, monocytes using the F4/80 antibody to murine mononuclear phagocytes, and osteoclasts by TRAP. **A to C:** Occlusal area. **D to F:** Basal area. Significance was demonstrated for both the occlusal ($n = 15$, $r = 0.76$, $P < 0.01$) and basal area ($n = 15$, $r = 0.548$, $P < 0.05$).

osteoclasts; in areas of bone formation there is a negative correlation.

It appears that there are two peaks of monocyte recruitment in the occlusal area occurring on days 5 and 9. Interestingly, Marks and Grolman have also noted a biphasic recruitment of monocytic cells to the occlusal area of the dental follicle during tooth eruption in rats.⁴⁵ This, coupled with the relatively low variability in each data point suggests that the biphasic response that we observed reflects a phenomenon that actually occurs in the occlusal area. We did not find conclusive evidence for a similar biphasic recruitment of monocytes in the apical area.

Discussion

Tooth eruption provides an opportunity to study developmental processes involving bone formation and bone resorption because it occurs predictably in a well defined area and is precisely regulated.³² We found that monocyte recruitment increased signifi-

cantly in areas where bone formation and bone resorption are developmentally regulated during molar eruption in mice. In occlusal areas, there was an association between the number of monocytes and osteoclasts, suggesting that monocytes may participate in bone resorption by fusing into osteoclasts or by secreting factors that facilitate this process. In the basal area, which differs metabolically from the occlusal, an increasing number of monocytes was not correlated with the osteoclastic activity; on the contrary, the number of osteoclasts declined with time. These results also suggest that monocytes are filling functionally different roles, as the bone in each area is undergoing physiologically distinct events. However, in both areas, a statistically significant correlation was observed between the recruitment of monocytes and the number of cells expressing MCP-1. MCP-1 expression has typically been linked to pathological processes and has provided a mechanistic basis to explain the recruitment of monocytes observed in chronic inflammation. This pattern of MCP-1 expression in tooth eruption represents one

of the first examples where expression of a chemokine is under developmental control. It is our hypothesis that MCP-1 expression is regulated during developmental processes that occur during tooth eruption, either directly in development or indirectly by other cytokines that are produced during this event. However, it remains to be established whether MCP-1 is the principal monocyte chemoattractant responsible for the recruitment of monocytic cells in this model.

Monocyte recruitment has been reported to occur in other developmental processes.¹ Based on the capacity of monocytic cells to exhibit phagocytic activity and to produce regulatory mediators, Morris and co-workers suggested that mononuclear phagocytes may participate in the remodeling of developing tissues. This may be particularly important in areas where extracellular matrix must be removed. An association between macrophages and programmed cell death has also been reported as a part of normal development. Abood and Jones² reported an increase in mononuclear phagocytes at sites of programmed cell death specifically associated in the developing rat diaphragm. Cuadros et al³ studied the embryonic avian central nervous system. Apart from a phagocytic role in areas of apoptosis, they found macrophages in intimate contact with growing axons and growth cones in axonal tracts at early developmental stages, suggesting that macrophages might be involved in axonal growth through release of biological mediators. Hopkins-Woolley⁴ conducted studies on mouse embryos and observed the recruitment of monocytes to areas of tissue remodeling in interdigital spaces during development of embryonic mouse limbs. This underscores the utility of recruiting phagocytic cells to participate in remodeling of extracellular matrix.

MCP-1 is a secondary inflammatory mediator, induced by primary mediators such as interleukin-1, tumor necrosis factor- α , interleukin-6, or interferon- γ .³³⁻³⁷ However, the stimulus for chemokine expression in noninflammatory conditions is unknown. Some consider an inflammation-like state a possible regulatory mechanism for ontogenesis. It is possible that primary inflammatory mediators are also developmentally expressed and would lead to the induction of chemokines such as MCP-1. This is supported by findings that expression of tumor necrosis factor- α is developmentally regulated.^{38,39} Thus, MCP-1 expression could be an important but secondary event stimulated by other mediators during developmental processes. This would be consistent with other cellular changes needed for recruitment of leu-

kocyte subsets, including the up-regulation of adhesion molecules on endothelial cells.

One surprising finding was that recruitment of monocytes occurred in areas associated with both bone formation and bone resorption. In areas of bone resorption, monocyte recruitment was directly correlated with osteoclast formation whereas in areas of bone formation there was an inverse correlation. This suggests that monocytes recruited to each area have functionally distinct roles. Monocytes in areas of resorption could produce factors leading to osteoclast formation and activation. For example, interleukin-1 and tumor necrosis factor- α produced by monocytes exhibit both properties and, in fact, are synergistic.⁴⁰ Furthermore, osteoclasts are formed from precursors of the monocyte lineage.^{41,42} Some of the recruited monocytes could potentially represent these precursor cells. In addition, monocytes could participate in removal of tissue through the production of lytic enzymes and phagocytosis. In contrast, monocytes in the basal area have no clear association with osteoclast activity. Monocytes recruited to these areas may have a different function and carry out regulatory activities specifically associated with bone formation or remodeling. In support of this concept, monocytes produce growth factors capable of stimulating osteoblasts such as platelet-derived growth factor, fibroblast growth factor, insulin-like growth factor-1, transforming growth factor- α , and transforming growth factor- β .⁴³ The potential for monocytes to participate in bone formation is supported by findings that a significant number are recruited to sites of bone injury and actively produce the above growth factors.⁴⁴ Whether or not these monocytes are functionally distinct requires further investigation.

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